Supporting Information

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SI Text

Demonstration of iPALM Resolution. An example of the 3 interference images of sparsely distributed fluorescent fiducials (100-nm Au particles chosen for 561-nm excitation and stability over the course of the experiment) at a specific z height is shown in Fig. S1*A*. These fiducials serve 3 purposes: to calibrate and align the system, to track any nanometer scale sample drift so that it can be corrected, and to demonstrate localization accuracy. If the sample is translated along the z axis, then the intensities cycle among the 3 cameras. Typically, we capture 800–1,200 photons total across all 3 cameras per frame for a single fiducial. A plot of the intensity vs. z position for a typical fiducial is shown in Fig. S1*B*. Using Eq. **3**, a z position can be extracted for each 3-element intensity vector. The z positions derived from the intensities for all fiducials (within field of 20×20 μm) imaged over a range of displacements is shown in Fig. S1*C* and demonstrates uniform sensitivity.

The observed spread of vertical locations within each frame is most likely dominated by variations in the fiducial heights across the field. Notice that the position can be determined uniquely in a 225-nm range which defines a preferred sample thickness. Thicker samples can be accommodated by incorporating an independent technique such as defocused PSF or the degree of coherence to determine the order of the interference fringe, an integer multiple of 225 nm.

One can test the variance in localization for a hypothetical source where ≈1,000 photons are captured, a value typical for a FP. This is emulated by repeatedly sampling (25,000 frames) a typical fiducial so that only 1,000–1,200 photons are captured per 50-ms sampling, and x, y, and z coordinates are calculated based on the data. A histogram of the position in Fig. 2*A* shows a variance of $\sigma_{xy} = 9.5$ nm laterally and a more precise $\sigma_z = 4.1$ nm using interferometry. The ratio $\sigma_z/\sigma_{x,y} \approx 0.43$ observed here is very close to the value of 0.40 obtained analytically from Eqs. **5** and **7**.

Experimental Setup. The experimental setup is shown schematically in Fig. 1*A*. The samples in study were sandwiched between 2 #1.5 coverslips (Warner Instruments). This assembly was placed between 2 opposing infinity-corrected microscope objectives (Nikon CFI Apochromat TIRF 60×, $NA = 1.49$, Nikon); index matching oil was used (Cargille type DF; Cargille Laboratories). We used the light from a 50-mW diode laser at $\lambda_{act} = 405$ nm (Coherent Inc.) for activation of the fluorescent proteins, and 150 mW, $\lambda_{exc} = 561$ nm diode pumped solid-state laser (CrystaLaser) for excitation of activated fluorescent proteins. Narrow-bandwidth laser line filters (MaxLine LL01–561-12.5 and MaxDiode LD01–405/10–12.5; Semrock Inc.) were used to reject both emission noise from the laser and autofluorescence generated in the optical path before the objectives. The activation and excitation beams entered the back pupil planes of the objectives through the slots in the custom turning mirrors (Reynard Corp.). The radial position of these beams was controlled to produce near-total internal reflection (TIR) condition. The fluorescence signal was collected by the infinity-corrected objectives and the image-forming beams were interfered in the 3-way beam splitter custom manufactured by Rocky Mountain Instruments. The output beams from 3-way beam splitter were then focused on 3 separate EMCCD cameras (Andor iXon DU-897; Andor Technology) via $f = 400$-mm achromatic lenses (01LAO799; CVI Melles Griot). To reject the excitation light from registration, we used the long-pass and band-pass optical filters (RazorEdge LP02–633RU-25 and BrightLine FF01–588/21–25; Semrock Inc.). A typical measurement consisted of 20,000–100,000 frame triplets with excitation/collection exposure time of 50 ms, and the 5- to 30-ms activation pulses were transmitted between the excitation pulses.

Sample Preparation. ***Coverslip preparation.*** First, the 18- and 25-mm #1.5 coverslips (Warner Instruments) were cleaned by immersion for at least 2–3 h in stirred solution at 85 °C of 5:1:1 H_2O/H_2O_2 (50%; Fisher Scientific)/NH_4OH (100%; Fisher Scientific).

After cleaning, 50 μl of 0.1% PolyL-Lysine (Ted Pella) was deposited on the top surface of the 18-mm coverslips and incubated for 30 min. The coverslips were then rinsed with H_2O and blow-dried with purified air.

After this, 50 μl of 5% (1:20 diluted in H_2O) Au 100-nm beads (100-nm Gold nanospheres 5.6E9 per ml, 20 ml; Microspheres-Nanospheres) was deposited on the top surface of 18-mm coverslips and incubated for 5 min. The coverslips were then rinsed with H_2O and blow-dried with purified air.

Finally, 50 nm of SiO_2 was deposited on the top surface of the 18-mm coverslips using Gatan 682 Precision Etching Coating System (Gatan) and cleaned again in the solution described above.

The cells were then grown on the top surface of the 18-mm coverslips. After fixation, the clean 25-mm coverslip was placed above the cells and sealed using 5-min epoxy (ITW Performance Polymers) and Vaseline (Unilever).

Mammalian expression vectors. All expression vectors were constructed using C1 and N1 (Clontech-style) cloning vectors. tdEos cloning vectors were prepared as previously described (1). A bacterial vector containing the coding sequence for mKikGR was amplified with a 5′ primer encoding an AgeI site and a 3′ primer encoding either a BspEI (C1) or NotI (N1) site. The purified and digested PCR products were ligated into similarly digested EGFP-C1 and EGFP-N1 cloning vector backbones. To generate fusion vectors, the appropriate cloning vector and an EGFP fusion vector were digested, either sequentially or doubly, with the appropriate enzymes and ligated together after gel purification. Thus, to prepare mKikGR-α-tubulin the following digest was performed: NheI and BglII (EGFP-α-tubulin ; Clontech), and to prepare tdEos-α-v-integrin the following digest was performed: AgeI and NotI (Waterman Lab Clontech-style N1-EGFP-α-v-integrin). DNA for mammalian transfection was prepared using the Plasmid Maxi kit (Qiagen). Vectors were verified by sequencing (FSU Biological Science Sequencing Facility) and were expressed in HeLa cells to confirm localization (Figs. S2 and S3].

To prepare the VSVGtsO45-NL-tdEosFP, the VSVGtsO45-NL-encoding DNA was removed from the VSVGtsO45-NL-dEosFP plasmid (previously described in ref. 2) by restriction endonuclease digestion using XhoI and BamHI and ligated into a similarly digested tdEosFP-N1 plasmid.

Cell culture and transfection. PtK1 (Potoroo kidney) cells (ATCC) (Fig. 3) were cultured in Phenol-red free DMEM-F12 supplemented with 10% FBS and 2 mM glutamine (Invitrogen). Cells were tyrpsinized by 0.25% trypsin/EDTA (Invitrogen), counted, pelleted, and resuspended in Nucleofector Solution R (Amaxa). Approximately 1×10^6 cells were mixed with 5 μg of mKikGR-tubulin and electroporated according to Amaxa Nucleofector protocol A-023.

Electroporated cells were plated onto iPALM coverslips that

had been washed for 1 h at 70 °C in 5:1:1 solution of H_2O/H2O2/NH4OH (see *Coverslip preparation*), washed in acetone, dried, baked at 120 °C for 1 h, then silanized with vapor phase HMDS (Hexamethyldisilazane; Sigma–Aldrich) as previously described (3), and then coated with 7.5 μg/ml human fibronectin (Chemicon) at 4 °C overnight. Coverslips were rinsed with supplemented media before the addition of electroporated cells.

COS7 (African green monkey kidney) cells (Fig. 4) were cultured in RPMI medium 1640 supplemented with 10% FBS, 100 mM sodium pyruvate, 100× non-essential amino acids, 100× MEM vitamin solution, 7.5% sodium bicarbonate solution, and 100 units/ml of penicillin/streptomycin (Invitrogen). Cells were trypsinized by 0.05% Trypsin/EDTA (Invitrogen) and cultured on iPALM coverslips 24 h before transfection. Cells were transfected using the Fugene 6 Reagent (Roche) according to manufacturer's protocol with a ratio of 3 μl of Fugene per 2 μg of VSVGtsO45-NL-tdEosFP DNA and fixed ≈24 h after transfection.

U2OS (human osteosarcoma) cells (Fig. 5) were cultured in McCoy5A media supplemented with 10% FBS, 2 mM glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen). Cells were grown for 2 days and trypsinized by 0.25% Trypsin/EDTA (Invitrogen), counted, pelleted, and resuspended in Nucleofector Solution V (Amaxa). Approximately 1×10^6 cells were mixed with 1 μg each of DNA vectors for tdEosFP-tagged α_v-integrin and untagged β_1-integrin (to aid in proper localization of the integrin $\alpha\beta$ heterodimer, obtained as a kind gift from Mark Ginsberg (University of California, San Diego, School of Medicine). Electroporation of the suspended cells were carried out using Amaxa nucleofector program X-001 per manufacturer's protocol. Electroporated cells were then directly transferred to 18-mm iPALM coverslips in a 6-well tissue culture plate. Phenol red-free McCoy5A media (HyClone) similarly supplemented, was used at this stage of culture to minimize any potential contaminating fluorescence from phenol red dyes.

To prepare for cell growth, iPALM coverslips were briefly rinsed with Dulbecco's phosphate buffered saline (DPBS; Invitrogen), and incubated with 10 μg/ml human plasma fibronectin (FC010–5MG; Chemicon International) at 4 °C overnight. Coverslips were then rinsed with DPBS and incubated with 1% heat-inactivated BSA (A3059; Sigma) for 1 h at 37 °C before a final rinse with DPBS.

Cell fixation. COS7 and PtK1 cells were treated with 10 nM taxol for 20–30 min at 37 °C before fixation to stabilize microtubules. All cells were fixed with either 2% paraformaldehyde (U2OS) or 2% paraformaldehyde and 0.05% glutaraldehyde (COS7 and PTK1) in PHEM buffer [60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgSO_4$ (pH 7.0)] at 37 °C for 10–15 min as previously described (4). All cells were stored in PHEM until imaged.

1. Shroff H, *et al.* (2007) Dual-color superresolution imaging of genetically expressed probes within individual adhesion complexes. *Proc Natl Acad Sci USA* 104(51):20308–20313.
2. Wiedenmann J, *et al.* (2004) EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proc Natl Acad Sci USA* 101(45):15905–15910.
3. Regen CM, Horwitz AF (1992) Dynamics of beta(1) integrin-mediated adhesive contacts in motile fibroblasts. *J Cell Biol* 119(5):1347–1359.
4. Galbraith CG, Skalak R, Chien S (1998) Shear stress induces spatial reorganization of the endothelial cell cytoskeleton. *Cell Motil Cytoskeleton* 40(4):317–330.

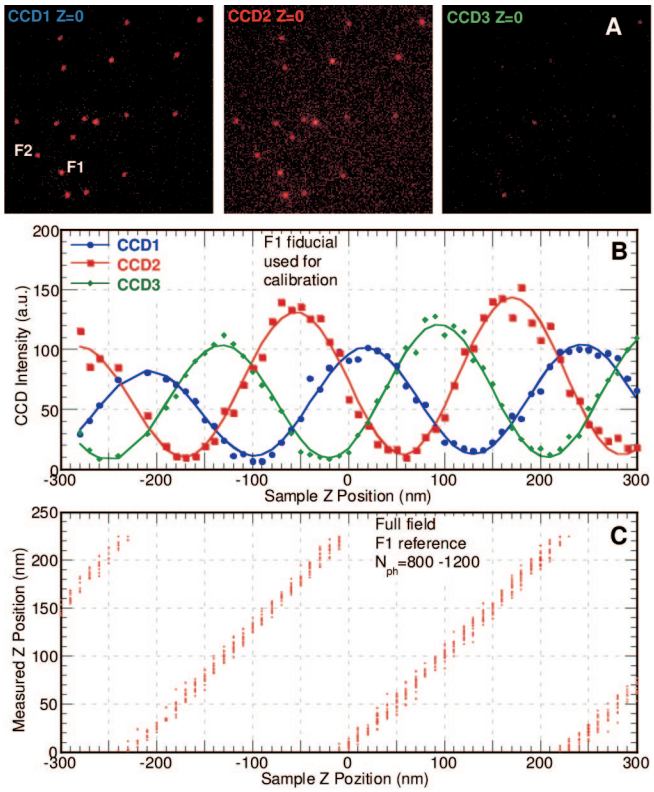


Fig. S1. Demonstration of interference on 3 cameras of reference fluorescent fiducials and resulting extracted *z* position. (*A*) Three different phase-interfered images of fiducials (100-nm Au particles). (*B*) The intensity oscillations of fiducial F2 for each of the 3 cameras as its vertical position is varied. (*C*) The extracted *z* position based the intensity triplet for all fiducials present in the image.

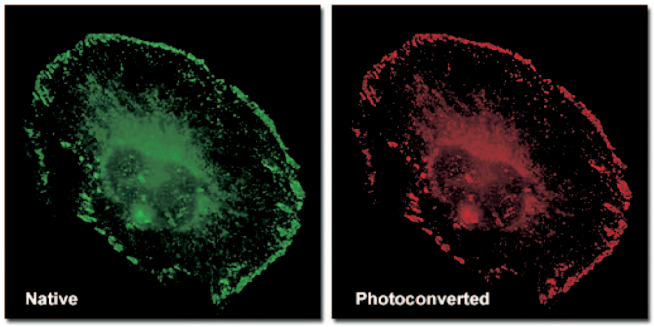


Fig. S2. tdEos-α_v-Integrin in HeLa cells.

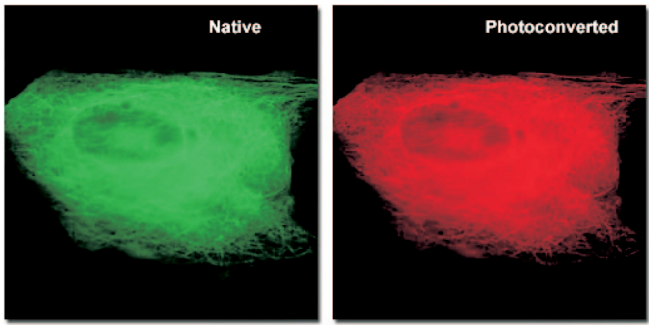
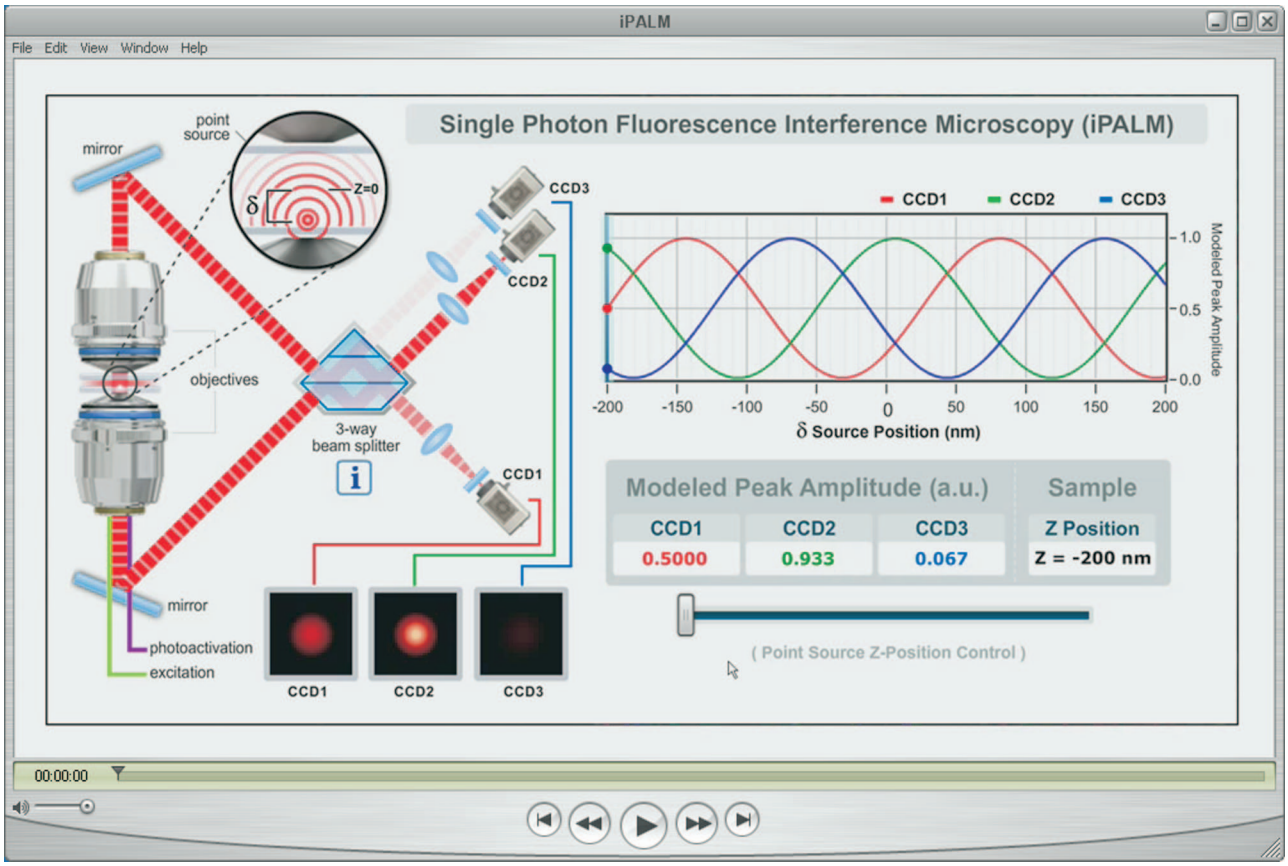
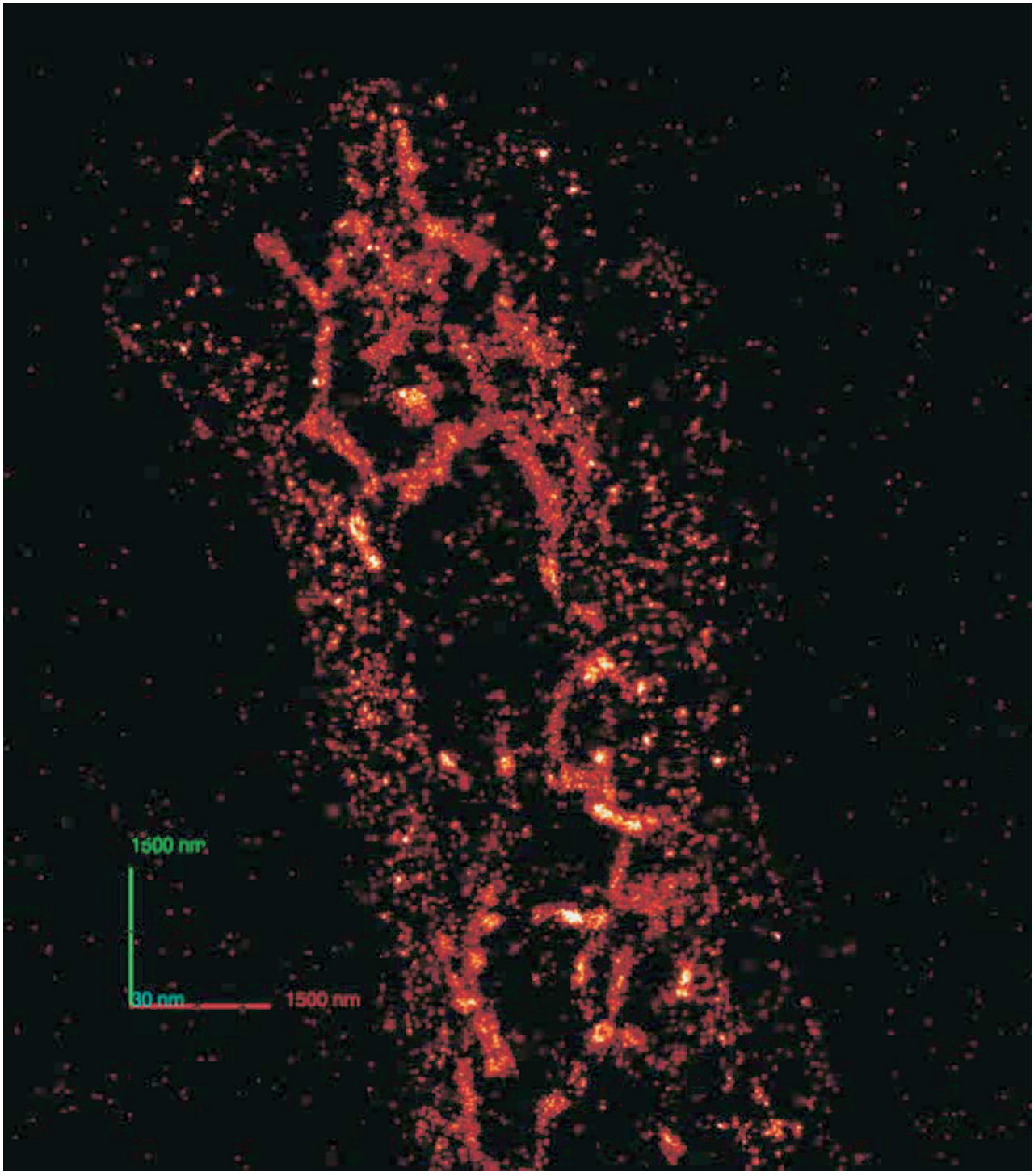


Fig. S3. mKikGR in HeLa cells.



Movie S1. iPALM tutorial, illustrating and dependence of the relative intensity of the interferometric image in 3 cameras on the vertical position of the fluorescent source.

Movie S1 (MOV)



Movie S2. iPALM-based 3D movie rendering of td-EosFP-α_v-integrin positions inside U2OS cell.

Movie S2 (MOV)